original research report

Preliminary report on tumor stem cell/B cell hybridoma vaccine for recurrent glioblastoma multiforme

Gustavo A. Moviglia,[a,b] Antonio G. Carrizo,[a] Gabriela Varela,[a] Carlos A. Gaeta,[a,b] Andrea Paes de Lima,[a] Pablo Farina,[a] Hugo Molina[b]

From the [a]Fundación Regina Mater, Buenos Aires, Argentina and the [b] CIITT from Universidad Maimónides, Buenos Aires, Argentina

Correspondence and reprint requests: Gustavo Antonio Moviglia, MD · Instituto Regina Mater · Paraguay 2452, Buenos Aires, 1121 ABL Argentina · T: +541149626800 ext 221 · F: +541149626800 ext 248 · gmoviglia@reginamater.com · Accepted for publication December 2007

Hematol Oncol Stem Cel Ther 2008; 1(1): 3-13

BACKGROUND: Glioblastoma multiforme (GBM), the most aggressive glioma, presents with a rapid evolution and relapse within the first year, which is attributed to the persistence of tumor stem cells (TSC) and the escape of immune surveillance. Mixed leukocyte culture (MLC) cytoimplant has been shown to function as a powerful intratumor pro-inflammatory cytokine pump. Tumor B-cell hybridoma (TBH) vaccines have been shown to function as antigen-presenting cells. We evaluated the toxicity and efficiency of each treatment alone and in combination.

PATIENTS AND METHODS: In an open study, 12 consecutive patients were evenly divided into 3 groups, each group receiving 3 different treatments. Patients in Group 1 were treated, after diagnosis, with debulking surgery (DS)+radiotherapy (Rx), and after the first relapse underwent DS+MLC treatment. Patients in Group 2 were similarly treated but after the first relapse underwent DS+MLC+TBH. Finally, patients in Group 3 were similarly treated but after the first relapse underwent DS+TBH. Nestin PAP stain assessed TSC participation in TBH.

RESULTS: Treatment with MLC had strong and rapid therapeutic effects, but was limited in duration and induced various degrees of brain inflammation. Treatment with MLC+TBH acted synergistically, provoking a rapid, strong and lasting therapeutic response but also generating different degrees of brain inflammation. A lasting therapeutic effect without generating high degrees of brain inflammation occurred in patients treated with TBH vaccine alone.

CONCLUSION: TSC vaccine consisting of TBH alone seems to have potent adjuvant reactions overcoming both persistence of tumor stem cells and immune escape of GBM without provoking an encephalitic reaction.

According to estimations made by the Central Brain Tumor Registry of the USA (CBTRUS), 17 000 people in the USA developed glioblastoma multiforme (GBM) during 2006.[1] GBM is the most aggressive stage of glioma. Even with the use of surgery and radiotherapy, this disease presents a rapid evolution and the first relapse occurs within the first year of diagnosis.[2-8] The maximum survival time observed for people after first relapse and with surgery plus temozolomide is about 8 months. This survival time presents a poor quality of life. Due to the rapid progression of the disease, the patient falls into a coma during the last months of its evolution.[2-8]

One of the modern theories of this relapse is attributed to the persistence of tumor stem cells that migrate towards healthy brain tissues. The tumor aggressiveness is determined not only by the high tumor growth rate, but by the local and systemic immune suppression induced by this tumor as well.[9-21]

Patients with GBM present with decreased cellular and humoral immunity. This is particularly observed through the analysis of a decreased response to common antigen skin tests (candidin, tuberculin, etc.), as well as the poor response of circulating lymphocytes to stimulation with phytohemaglutinine (PHA). The production of antibodies to the tetanus toxin and to the flu vaccine is also diminished.[16-21]

This deficiency in the general immune response is thought to be induced by antinflammatory tumor factors, mainly by transforming growth factor b (TGFb).[16,17] The specific lack of immune response against GBM is attributed, first, to the above described general immune

deficiency, and second, to the tumor cell lack of the major histocompatibility complex (MHC)[18,19] and costimulatory molecules. Both phenomena interfere with the recognition of tumor cells as malignant cells by CD4 and CD8 lymphocytes.[16-21]

Over the last twenty years, diverse immunotherapy protocols have been developed. As common features, these treatments involve the surgical reduction of tumor mass followed by local or systemic treatment with active or passive immunotherapy, non-specific stimulants (BCG vaccines, *Corynebacterium parvum*, and others), LAK cells implants, TIL cells, modified lymphocytes or fibroblasts associated or not with leukins or other biological response modifiers, monoclonal antibodies alone or in conjunction with drugs. Despite the good results obtained in animal models, few of these approaches have produced truly significant results in clinical trials.[21-43]

Since 1997, our group applied, under a compassionate basis, two of these protocols: 1) The cytoimplant of a mixed lymphocyte culture (MLC) in the tumor lodge, a technique developed by Granger,[44] discoverer of tumor necrosis factor and 2) systemic vaccination with a glioma cell B-lymphocyte hybrid (TBH). Each therapeutic procedure was applied separately or in association.[45,46] These methods were chosen based on previous animal experience, as well as our own therapeutic results, and those observed by other researchers in the treatment of human pancreatic cancer,[44,47] a tumor that presents with similar immune features: high aggressiveness related to the high production of TGFb and the tumor cell lack of MHC molecules as well as their corresponding costimulatory molecules.

Chang et al used MLC cytoimplant to treat stage I-II pancreatic cancer[44] with promising results. Its mechanism of action is attributed to the production of a strong primary graft vs. host rejection which begins to operate as an intratumor powerful TH1 leukin pump.[44,47,48] A TBH vaccine was used to treat different tumors. This is an autologous cell hybrid formed by the fusion of autologous tumor cells with autologous B-lymphocytes. Vaccination with this cell hybrid produces a specific antitumor reaction, which takes 45 to 60 days to be detected in the blood.[45-49]

As was proved for the treatment of pancreatic cancer and breast cancer,[47,48] the association of MLC and TBH can elicit a synergistic effect improving their therapeutic outcome. The explanation for this synergistic effect can be summarized as follows: the action of TBH is promoted by the leukins generated by the reaction of the MLC, which also shortens its reaction time, generating a stable effector response (TH1).

The present report summarizes our experience using these two methods (as a single biologic agent or a combination of both), with special emphasis on their feasibility, toxicity and possible therapeutic benefits.

PATIENTS AND METHODS

The patients included in the present study, who were recruited between 1997 and January 2004, were males or non-pregnant females with a positive diagnosis of single and completely resected first or second relapsing GBM. The positive GBM diagnosis was performed by an experienced neuropathologist after the analysis of the resected surgical piece. The pathological analysis of the surgical piece met the three morphological criteria established by the WHO: a high degree of gliomorphic cytological malignancy, necrotic areas and high vascularization of the central part of each live cell tumor mass (Figures 1, 2).[2,3] In addition, vimentin and glial fibrilliay acidic protein (GFAP) (Zymed, San Diego, USA) immunoperoxidase stain were positive (Figure 3, 4). As an additional study, a nestin (R&D System, Grand Island, USA) immunoperoxidase was performed to assess the presence of tumor stem cells.[9]

Previous treatment of the primitive tumor included total surgical resection and a complete 3D radiotherapy scheme. At the beginning of treatment, the patients' clinical performance met the ECOG 0-3 criteria[40] and the spectroscopic analysis of the postsurgical MRI showed no remaining tumor mass. These patients had not received radio- or chemotherapy for at least a month prior to initiation of their immunotherapy regimen. Immune, cardiac, hepatic, respiratory and renal functions were preserved and there was no second active neoplasias, and no active viral or bacterial infection or visceral mycosis at the time of admission to the clinical trial. All patients provided signed, informed consent.

Twelve consecutive patients were evenly divided into 3 groups, each receiving a different treatment. The first four patients (Group 1) were treated, after diagnosis, with debulking surgery (DS)+radiotherapy (Rx) and after first relapse, they underwent DS + MLC treatment. The next four patients (Group 2) were similarly treated but after first relapse underwent DS+MLC treatment+TBH vaccination. The last four patients (Group 3), after the first relapse were similarly treated, though they only underwent DS+TBH vaccination.

MLC treatment: In this procedure, which has been previously described,[44,47,48] a non-related donor was selected and peripheral mononuclear cells (MNC) were collected from the donor and patient by apheresis (Cobe Spectra, Chicago, USA). MNC were purified and washed; the patient's MNC were irradiated and used as a challenge to activate donor MNC during a

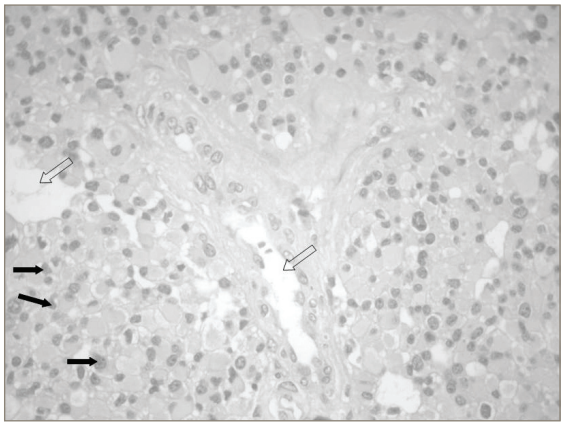

Figure 1. High magnification of recurrent glioma anatompathologic study from patient shown in Figure 7. Solid arrows show different nuclear shapes characteristic of this highly gliomorphic cytology. Empty arrows show the high degree of vascularization present in those GBM patients. (H&E stain).

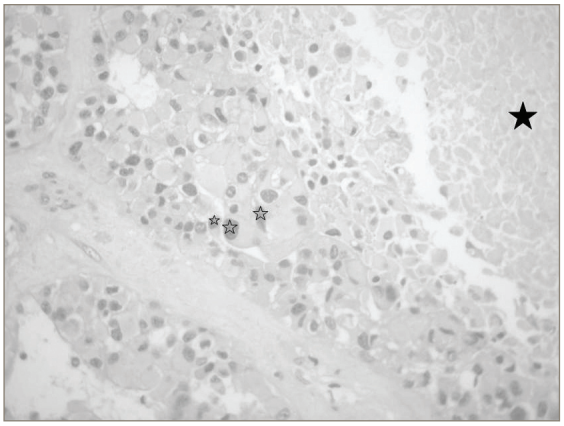

Figure 2. White stars show highly gliomorphic cytology (gliomorphic cytologic malignancies). Black stars show necrotic areas from the same biopsy described in Figure 1.

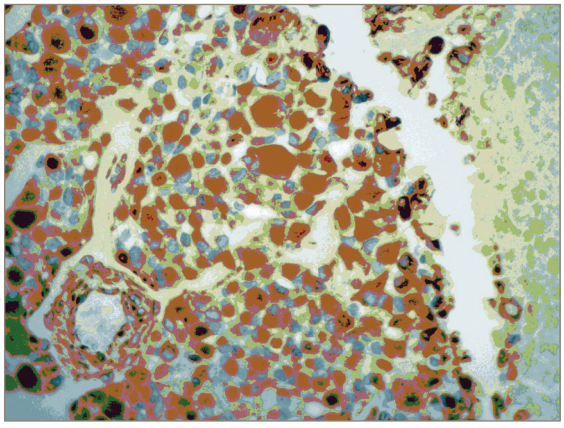

Figure 3. Brown spots show vimentin PAP positive stains of the same biopsy described in Figure 1. Arrows show positive stain spots.

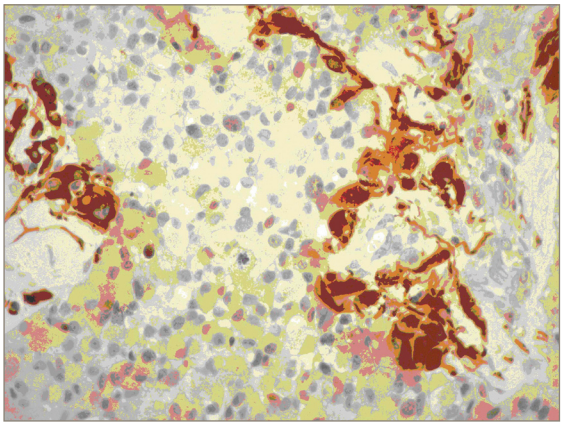

Figure 4. Glioblastoma multiforme (GBM), glial fibrilliay acidic protein (GFAP) PAP immunostain.

three-day mixed leukocyte culture. The activated cells were implanted into the tumor lodge after the neurosurgery in a fibrin cloth performed with a commercial kit (Tissucol, Baxter-Immune, Chicago, USA).

The tumor specimen extracted during surgery was divided into two parts, one part used for pathological studies and the other, processed as an antigen. The half tumor sample processed as antigen was mechanically dissociated to a single cell suspension. This cell suspension was washed, the gradient purified and seeded in DMEM (Gibco, Grand Island, USA) enriched with insulin and epidermal growth factor. Non-fetal calf serum or human serum was added to this culture medium.

Development of TBH: In this procedure, which has been previously described,[46] the patient's mononuclear lymphocytes were obtained by apheresis (Cobe Spectra, Chicago, USA). B cells were purified from this mononuclear cell sample by negative selection (Stem Sep Kit, Stem Cell Technology, Vancouver, Canada). The B cells were cultured in a DMEM (Gibco, Grand Island, USA) enriched with IL6 (Stem Cell Technologies, Vancouver, Canada) and IL4 (Stem Cell Technologies, Vancouver, Canada) medium for three days. Tumor cells were fused with activated B-cells using PEG (Merck, Munich, Germany), thereby generating a TBH autovaccine. The immunizations were given by intra-lymph node injection every three weeks upon medical indication.

For quality control, microbiological studies were

conducted on samples taken from apheresis products, from selected cells, and from the final product of each culture to detect contamination by bacteria, fungus or virus. If any sample proved positive, the final product was treated with antibiotics and/or antimycotics according to the in vitro sensitivity of the contaminants until negativization of culture; otherwise it was discarded and the procedure repeated. A double immune stain was performed with anti-CD 19 and anti-vimentin to corroborate that the hybrid cell had been formed. A nestin (R&D Systems Inc.) immunoperoxidase stain was performed to assess the contribution of tumor stem cells as a component of the TBH hybrid.

Clinical controls and follow-up: During the week previous to any treatment, the patients went through several controls: a complete clinical history with particular emphasis on the neurologic examination, clinical analyses, electrocardiogram, and pulmonary function, as well as immune analyses. The ECOG index was established according to international standards.[49] An MRI of the brain was also obtained.

Patients were controlled by the same physician once a week during the first trimester of treatment, every fifteen days for the following six months, and after that, once a month until the end of two years of treatment. At each examination, the clinical history was updated and lab tests were performed. The immune response against the tumor was analyzed prior to each vaccine; then, after the vaccination plan was completed, the immune response was analyzed every month for the first year, and every three months thereafter. The data regarding toxic reactions were specially reported to the safety committee. Patient survival was monitored monthly.

Criteria of tumor response: The two main criteria were survival, evaluated by telephone communication once a month, and tumor mass presence, assessed at 6 months post beginning of relapse treatment (MLC cytoimplant for Groups 1 and 2, or first TBH vaccine immunization for Group 3). For the second criterion, three degrees of response were established: 1) complete remission (CR): no detection of any tumor mass assessed by MRI and spectrometry analysis of MRI images; 2) partial remission (PR): minimal tumor mass present at the borders of the original tumor lodge (the total tumor mass present at evaluation time should be smaller than 50% of the original tumor mass); and 3) progressive disease (PD): at the evaluation point there was a relapse of the original tumor mass or new metastasis in the CNS larger than 50% of original tumor mass. Patients who presented as CR or PR were considered "responders" and those who presented as PD were "non-responders".

Evaluation of toxicity: Adverse events were evaluated by the "Common Terminology Criteria for Adverse Events (AE), 2004" developed by NIH. The definitions are available on the web at http://ctep.cancer.gov/reporting/ctc.html.

Immune evaluation: The immune response against the tumor was evaluated through a DTH reaction and the lymphocyte proliferation index (LPI).[50] The DTH reaction is an intradermal reaction performed using 105 irradiated autologous TBH cells. Cells were suspended in 0.1 cc buffer salt solution (BSS) and intradermally injected. As controls, 105 irradiated heterologous TBH cells were used. For the LPI,50 the MNC taken from a 20 cc blood sample from the patient were layered in a Ficoll-Hypaque gradient (Beckman Coulter Inc., Fullerton, CA, USA) and spun for 10 minutes at 300 G. These cells were seeded in a 96-well plate in TC 199 to which different immunogenic substances were added: 1) MNC without stimulus; 2) MNC without stimulus + patient's serum; 3) MNC vs. patient TBH cells, and 4) MNC+patient serum vs. patient TBH cells. Each assay was carried out in duplicate with 5×103 MNC seeded in each well. After 96 hours of culture, the total number of cells was counted using an automatic cell counter.

LPI was calculated as the rate between the number of challenged MNCs over the number of nonstimulated MNCs. If the index was lower than 0.7 the immune system tolerated the tumor. If this index proved higher than 1, the immune system had developed an antitumor effector reaction against the tumor.

Statistical data analysis: The Kaplan-Meyer test was used to evaluate the data on survival. The tumor response, immune response, and toxicology data were analyzed using analysis of variance. We looked for *P* values lower than 0.01 as statistically significant.

RESULTS

We recruited four patients for each group. Clinical conditions and treatment results are summarized in Table 1.

GBM cell and TBH culture: Contrary to what occurs in most primary cultures obtained from surgical pieces of carcinomas and sarcomas, the mechanical dissociation of the GBM tumor mass produced an almost pure suspension of malignant cells characterized by nuclear and cytoplasmic polymorphism. After about 15 days, the number of cells in culture experienced a dramatic drop of about 97% to 99% from the initial amount. These remaining cells were able to grow in a sustainable manner and established in culture in all 12 of the treated patients. These cells grew during 15 days until a critical tumor cell mass was obtained to be hybridized

Table 1. Clinical conditions and results.

Patient	Age	Sex	Second Surgery	Chemotherapy	Radiolotherapy	ECOG	Therapy	Survival after recurrence (months)	Dead
Li	49	F	yes	1	1	3	MLC	2	yes
Ey	51	M	yes	0	1	2	MLC	6	yes
Sa	46	M	yes	0	1	1	MLC	15	yes
Mo	65	F	yes	0	1	2	MLC	3	yes
Sr	36	M	yes	0	1	1	M+T	6	yes
Ar	71	F	yes	0	1	3	M+T	8	yes
Pi	58	M	no	0	0	1	M+T	15	yes
Vi	52	M	yes	0	1	2	M+T	7	yes
Va	51	M	no	0	1	1	TBH	18	yes
Rz	72	M	yes	1	1	3	TBH	14	no
Gz	48	F	yes	0	1	1	TBH	25	yes
Ss	33	F	yes	0	1	1	TBH	37	no

MLC: mixed leucocyte cytoimplant; TBH: tumor B-cell hybridoma vaccine; M+T: MLC+TBH. For chemotherapy, 1=complete standard therapy, 0=no chemotherapy. For radiotherapy, 1=complete dose of radiation, 0=no radiotherapy.

with autologous B lymphocytes of the patient.

The yield of this fusion was 14%, with respect to the original amount of cells used. This cellular population proved to be homogeneous, with monomorphic and lateralized nuclei and large star-shaped cytoplasm (Figure 5). The primary double immune staining of these cells with anti-CD19 and anti-vimentin allowed us to establish its hybrid character. Afterwards, four of these cell lines preserved in culture were stained with nestin, all being positive for this staining, hence certifying the GBM-stem-cell origin of this hybrid (Figure 6).

Immune response: For patients in Group 1, the MLC cytoimplant generated a rapid effector response 15 days after the procedure was completed, in the responder as well as in the non-responders. This response lasted until the fourth month in the responder patients, and stopped at three weeks in non-responder patients. In two patients, we observed a clear autoimmune response that generated encephalitis degree 2 according to the "Common Terminology Criteria for Adverse Events (AE), 2004" developed by NIH.

For patients in Group 2, the MLC cytoimplant followed by six immunizations with TBH respectively produced a marked effector in vitro response 15 days after the completion of the cytoimplant. In the responder patients, the response continued, increasing steadily for the duration of the entire treatment. In these patients, the autologous serum continually acted as a promoter of the in vitro effector reaction. Although all the patients treated were responders, two showed a remarkable encephalitic autoimmune reaction above degree 3-4 that obliged us to suspend the MLC treatment.

For patients in Group 3, the six immunizations with the TBH autovaccine showed in the four responder patients the appearance of an effector reaction in the peripheral lymphocytes around the third month of treatment. This response lasted for three more months. The presence of autologous serum showed a dual effect. During the first three months, it had inhibitory effects on the proliferation assay. After this 3-month period the patients developed an effector response. The expected autoimmune inflammatory reaction was degree 1 in 1 of 4 patients and absent in 3 of 4.

Tumor response and survival: In Group 1 (treatment with MLC alone) 3 of 4 were responders (CR+PR) and 1 of 4 had progressive disease (PD). The median survival of this group after MLC treatment was 4.5 months. In Group 2 (treatment with MLC and six immunizations with TBH), 4 of 4 were responders (CR+PR). The median survival of this group was 9.5 months after MLC treatment. In Group 3 (immunization with autovaccine TBH alone), 3 of 4 were responders (CR+PR). The median survival of this group was 25 months after the first immunization. The chi-square was 2.87 and had a significance level of P=0.11. (Figures 7, 8, 9 and 10)

Adverse events and side effects: Patients in Groups 1, 2 and 3 developed flu-like symptoms and hyperpyrexia

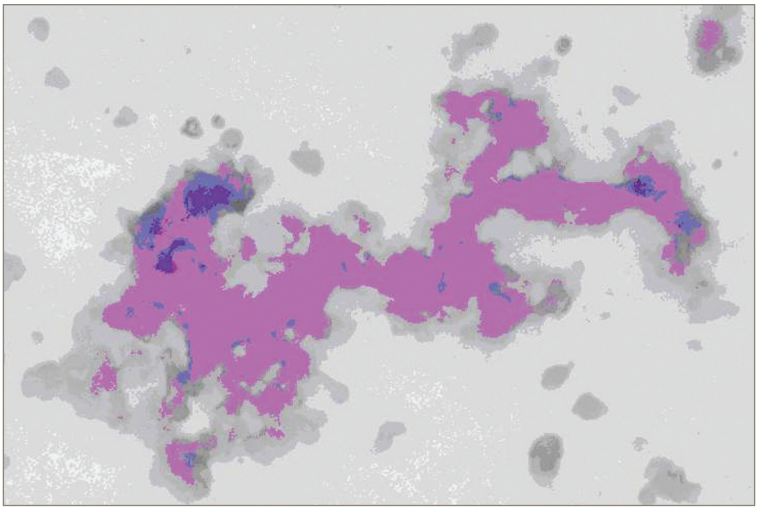

Figure 5. Single TBH cell in tissue culture, Giemsa stained.

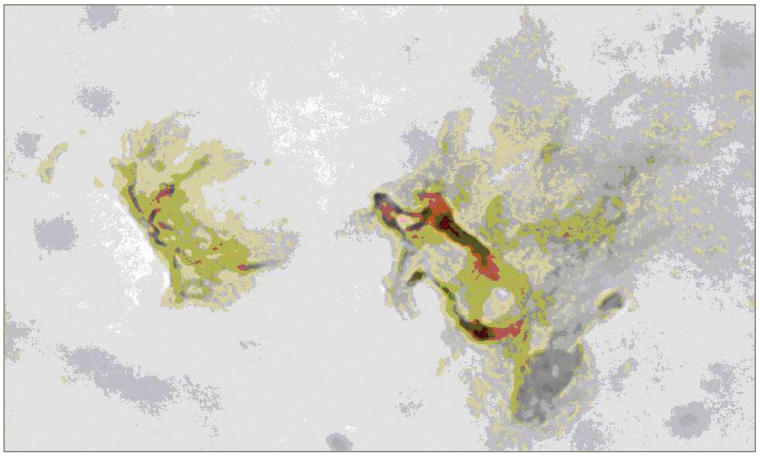

Figure 6. Note the cytoarchitectural stain of nestin positive reaction inside two large hybrid cells.

Figure 7. MRI of pre-operated recurrent GBM patient of group 3 (treated with TBH alone).

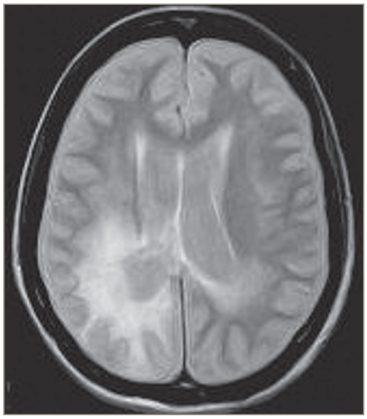

Figure 8. MRI of patient in Group 3 (treated with TBH alone) three years after treatment.

from 37.5 to 39.5ºC during the first 24-72 hours post-vaccination (Table 2). The severity of the fever did not seem to have a direct correlation to the favorable prognosis of the therapeutic response. Eight of 12 patients presented with nausea or gastrointestinal upset associated with the use of non-steroidal analgesics. These symptoms were resolved either by discontinuing the medication or combining it with metoclopramide. Of the patients in Groups 1, 2 and 3, 2 of 4, 4 of 4 and 1 of 4, respectively, experienced transitory episodes of hypotension during the first 60 hours after the cytoimplant or the immunization with TBH. The patients required no therapy. Encephalitic autoimmune reactions were present in 50% of the patients treated with MLC cytoimplants (Group 1, 2 of 4 and Group 2, 2 of 4). No cardiac, respiratory, kidney, blood toxicity or clotting disturbances were observed in any group.

DISCUSSION

According to Gatcombe[8] "patients diagnosed with glioblastoma multiforme (GBM) have a notoriously grim prognosis; median survival is only 13 months with multimodality treatment.[4] The current standard of care for these patients is surgical resection followed by concurrent radiation and temozolomide (TMZ), and then by adjuvant TMZ. This regimen has been demonstrated to improve median survival by a modest 2.5 months.[5] Thus, new interventions are desperately needed for patients diagnosed with GBM."

The discovery of the high sensitivity of GBM to epidermal growth factor (EGF),[21-24] brought the use of 13-cis-retinoic acid, which is an inhibitor of the EGF receptor transduction.[22] The good results observed led to the use of monoclonal antibodies (MAB) directed against this receptor.[23,24] Both treatments produced an effective increase in the survival rates of GBM patients. This approach only applies to newly diagnosed patients and its action is limited in time and seems to extend but not prevent the relapse of the tumor.

The fact that GBM cells present clearly identifiable antigen molecules, which may be the target of an immune system attack, inspired several immunotherapy approaches. These antigens, called tumor-associated antigens (TAA), are molecules different from the normal structures or expressed in an abnormal amount or corresponding to the structure of an age different from the individual's chronological age.[18]

Different active and passive immunization techniques have been successfully used in animal models. For years, however, these techniques failed to produce the same successful outcome in human trials.[16-43] The high levels of transforming growth factor b (TGFb)

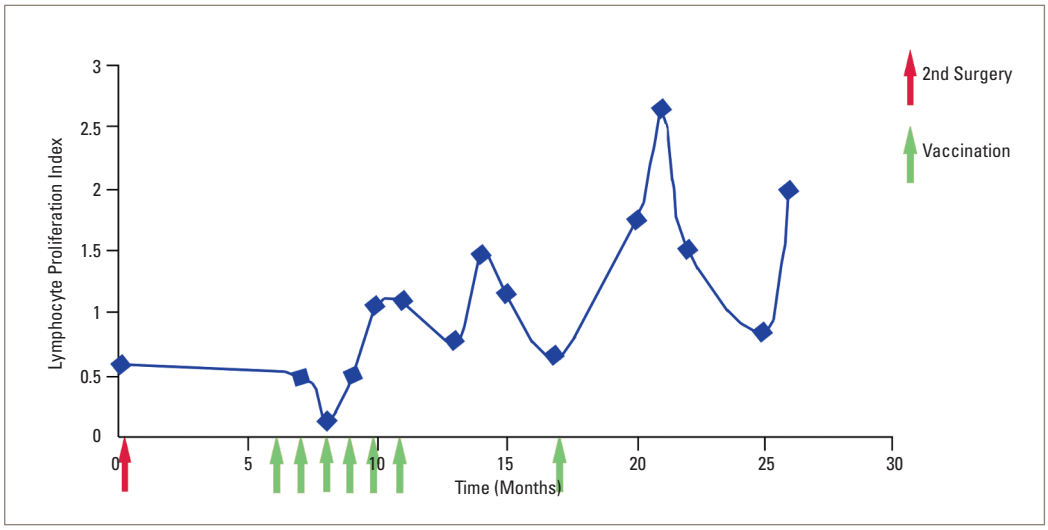

Figure 9. Immune evolution of lymphocyte proliferation index analysis of Figure 1 patient.

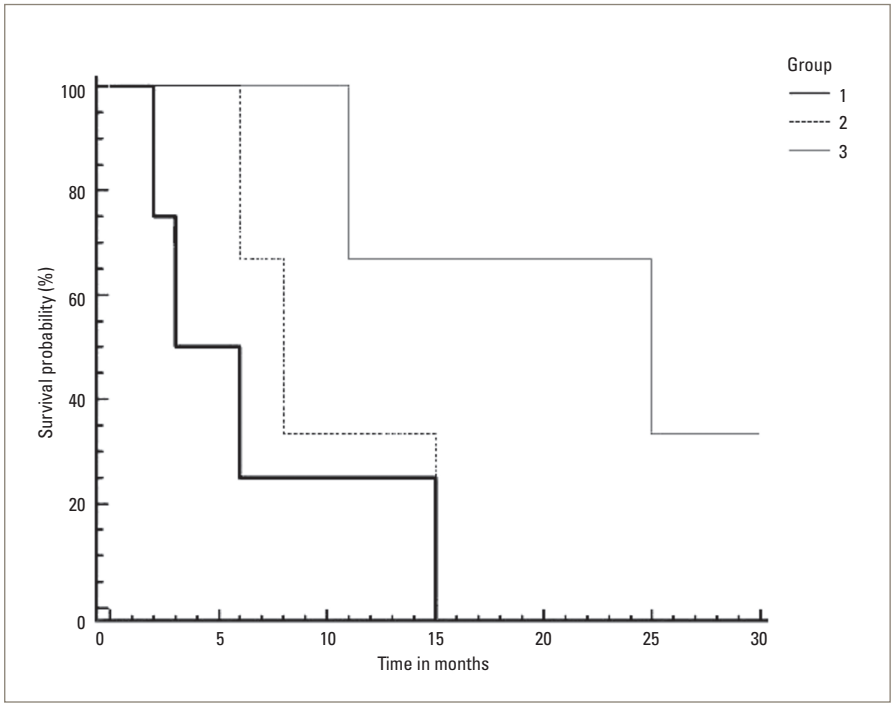

Figure 10. Kaplan-Meyer analysis of GBM-relapsed patients after different treatments. Group 1: MLC treatment; Group 2: MLC + TBH; Group 3: TBH.

produced by this tumor and the poor expression of co-stimulatory molecules on GBM cell membranes seem to be the major causes of immune therapy's failures.[16-20]

TGFb is the most important secretion of GBM. This cytokine causes immune suppression, typical of GBM, as well as inductions of immune tumor tolerance.[16-18] The surgical removal of the TGFb source supports these facts. Patients may show a recovery of their immunity during the first month postsurgery.

Using the therapeutic window of postsurgical immune recovery, several adoptive immunotherapeutic approaches have been used. There are therapies aimed at enhancing the local immune status[26-36] by the infusion of lymphokine-activated killer (LAK) cells or cytokines,

Table 2. Adverse events in the 12 patients included in the study.

	Toxicity							
Patient	Flu-like Syndrome	Brain immflammation	Kidney	Lung	Cardiovascular	Gastrointestinal	Hematology	Others
Li	0	0	0	0	0	0	0	0
Ey	0	2	0	0	0	0	0	0
Sa	0	2	0	0	0	0	0	0
Mo	1	4	0	0	0	0	0	0
Sr	1	4	0	0	0	0	0	0
Ar	1	0	0	0	0	0	0	0
Pi	1	2	0	0	0	0	0	0
Vi	0	2	0	0	0	0	0	0
Va	0	1	0	0	0	0	0	0
Rz	1	0	0	0	0	0	0	0
Gz	1	1	0	0	0	0	0	0
Ss	0	0	0	0	0	0	0	0

Numbers indicate toxicity degree, where 0 represents no adverse event observed; 1-2 means mild adverse events and 3-4 means important adverse events observed.

such as interleukin-2 (IL-2)[26-32] or interleukin-12 (IL-12)[33], or the infusion of granulocyte macrophage colony-stimulating factor (GM-CSF)[34,35] in the tumor lodge after a debulking surgery. However, these techniques were reported to have transient effects and to produce toxic side effects.

Granger's team developed an adjuvant treatment based on the implant of a mixed culture of leucocytes (MLC) in the tumor lodge (cytoimplant).[44] This MLC was generated by mononuclear cells (MNC) of an unrelated donor against the patient's inactivated MNC. MLC cytoimplant produced a powerful Th1 leukin pump in the surgical lodge that, due to the special characteristics of the GBM and the brain, was surrounded by tumor cells, which diffusely infiltrate the normal tissue. The Th1 pump favored the access of the patient's effector lymphocytes, which learned how to act not only against the heterologous tumor graft, but also against the remaining tumor cells.[44,47,48] In an early trial using this approach, 2 of 9 recurrent GBM patients achieved 3 years disease-free survival. This therapeutic approach was also applied in patients with stage II pancreatic tumors achieving similar successful results.[44]

Based on the presence of TAA and the poor costimulatory expression of GBM cell membrane, several vaccine protocols were developed.[36-43] They used peptides, dendritic cells or irradiated cells either alone or in combination with dendritic cells to improve the antigen presenting function of the tumor antigen.

Peptide vaccines induced specific antigen immunity against specific TAA. There was poor or no cross reactivity against other TAA elicited by this type of vaccine.[36-43] Moreover, those antigens belonged to differentiated forms of the GBM cells but were not present on the tumor stem cells.[8-15] Therefore, the GBM stopped producing cells to carry on those antigens and developed new tumor cells free of TAA1. This biological condition was named "tumor editing".

To avoid tumor editing, new vaccines used whole tumor cells. Three main approaches have been used. First, dendritic cells boosted with whole tumor cells,[36,37] GBM lysate,[38] several specific membrane antigen[39,40] or tumor nucleic acids.[41] These techniques were applied with relative clinical success, but only had positive therapeutic effects in newly diagnosed patients. A second approach was the use of genetically modified tumor cells producing an effector that induces leukins[35]; and third, dendritic GBM-fused cells.[43] Those approaches showed some extra effectiveness as compared to the peptide vaccine approach but, even when they prolonged disease-free survival time, they failed to prevent a relapse. The main cause of this failure (relapse prevention) may be attributed to the lack of effectiveness to elicit an antitumor stem cell reaction which, in time, developed a more primitive undifferentiated GBM lacking the previously showed TAA as well as remaining chemoresistant.[11-14]

In conjunction with these therapeutic developments, in 1996 our group started to use Granger's technique to treat GBM patients. After the first four treated patients, this therapy was combined with a tumor vaccine we have developed in an attempt to improve the response rate of the MLC cytoimplant.[45,46] As mentioned above, either the poor presence or the lack of costimulatory molecules of the tumor cells induces tumor immune tolerance.[18-20] To overcome this problem, we used a tumor hybrid vaccine produced by the fusion of the patient's tumor cells and autologous activated B cells (TBH). Tumor cells provide the TAA and the B cells provide MHC I and MHC II, costimulatory and adhesive molecules.[45,46] This hybrid of fused cells vaccine, unlike the dendritic GBM fused cells vaccine, can grow in vitro for a long time, allowing patient immunization with the same cell in a sufficient amount for the whole vaccination program.[43,45,46]

Chen et al. showed that TAA are present in GBM tumor stem cells.[15] We hypothesize that, as was reported, even when TAA are weak, when presented in a TBH cell shape, they could be easily recognized by the lymph node dendritic cells, thus generating a specific cytotoxic reaction with effective antitumor activity.

Comparing the therapeutic and the immune results described in the corresponding section, we observed that the MLC treatment itself produces strong and rapid therapeutic effects. However, its action seems limited in time and may provoke an important, hard to treat, autoimmune reaction against healthy brain parenchyma. Combining this treatment with TBH vaccine prolongs the immune activity against the glioma but, again, the patient could suffer an autoimmune reaction in the brain.

The inflammatory response observed in 4 of 8 patients in both groups (Groups 1 and 2) seemed to be the mainly responsible for the failure of the long-term duration of the earlier obtained successful results. This brain inflammatory response was attributed to an unwanted side effect of the MLC cytoimplant. Therefore, it was decided to treat a third group only with 6 TBH immunizations after the debulking surgery of relapsed GBM. The immune response of TBH alone seems to be slower than either MLC alone or combined with TBH but seems to be more effective and less toxic.

The quality and extent of the autoimmune inflammatory reaction observed in GBM patients treated with MLC (alone or in combination with TBH), is more significant than that observed in patients with other solid neoplasia treated with the same therapies. Pancreatic and breast cancer patients treated using a combination of MLC and TBH presented better tumor response and survival rates than those treated with either therapy alone.[47,48] Moreover, in Groups 1 and 2, MLC alone or MLC combined with TBH, seemed to have no statistically different effect on the survival time, in contrast with the longer survival time observed in Group 3, which only received TBH.

The outstanding results obtained with the use of TBH alone, compared with other pathologies, led to the idea that the greater success in the use of TBH was associated with the use of a tumor cell subsets. This assumption was reinforced by the particular behavior exhibited by these cells in tissue culture. After the GBM tissue dissociation, a large number of polymorphic tumor cells were obtained. This cell culture suffered a dramatic drop in its number. Only about 1% to 3% of the seeded cells survived. Surviving cells were a homogeneous mass with monomorphic and lateralized nuclei, and with a star-shaped cytoplasm. The culture conditions lacked substances commonly used by other researchers such as fetal calf serum and fibroblast growth factor. It consisted only of DMEM medium enriched with human insulin and human epidermal growth factor without any faetal calf serum or human serum supplementation. According to the work of Singh et al.,[9,10] this growth pattern is proper for GBM stem cells.

At the end of the 1990s, tumor stem cells were identified as tissue cells in which those mutations necessary for the transformation of stem cells into tumor cells take place. They could, in experimental models, reproduce the total tumor mass while the rest of the GBM cells were unable to do so. For this reason they are considered responsible for tumor relapse after a successful debulking surgery. Moreover, the GBM stem cell population is particularly resistant to chemotherapy and radiotherapy.[9-15]

Since this is a primitive cell, its antigenicity is different from that presented by the more developed stages of the tumor from which this cell originates. For this reason, those vaccines developed against the differentiated forms of a tumor are mostly effective for the control of these forms, but ineffective to attack the tumor stem cells and the mutated clones which differentiate to the previously mentioned forms.

Singh et al[9] have determined that there are two characteristic GBM stem cell markers: CD 133 and nestin molecules. The first is a membrane protein and the second is a component of the neural stem cell and GBM stem cell cytoskeleton. Neither molecule is present in the differentiated stages of the tumor cells or in normal nerve cell tissue. The fact that hybrids react positively to nestin proteins allowed us to believe that the TBH cells are formed by tumor stem cells. The particular

pattern of GBM cells observed in our cultures is associated with a positive nestin stain observed in TBH. According to Singh et al.,[40] both are univocal characteristics of GBM stem cells and support our assumption that this particular TBH is formed by GBM stem cells. Our results suggest, as implied by other authors, that GBM stem cells may be used as a basis for a tumor vaccine.[10,14,15]

In conclusion, a TBH GBM stem cell-based vaccine seems to elicit a specific tumor immune reaction without harming normal nerve tissue. This immune reaction seems to be strong enough to control or destroy the growth of the remaining GBM stem cells diffused in the normal brain tissue, which is supported by the outstanding disease-free survival time with a minimum of toxicity observed in patients of Group 3. A larger trial is in progress to assess the veracity of these preliminary conclusions.

Acknowledgements

In memory of Dr. Molina H. The critical review of Mark Renneker MD and Henry Smilowitz PhD, the spiritual support of Ms Maricel Brandolino and Dr. Ernesto Goverman, the technical assistance of Ms Carolina Beascoechea, and the economic support of Fundación Regina Mater are gratefully acknowledged.

REFERENCES

1. Central Brain Tumor Registry of the U.S (CBTRUS) data 2005
2. Burger PC, Vogel FS, Green SB, et al. Glioblastoma Multiforme and anaplastic astrocytoma: Pathologic criteria and prognostic implications. Cancer 1985; 56:1106-1111
3. Nelson JS, Tsukada Y, Schoenfeld D, et al. Necrosis as a prognostic criterion in malignant supratentorial, astrocytic gliomas. Cancer 1983; 52:550-554
4. LaCroix M, Abi-Said, Tourney DR, et al. A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival. J Neurosurg. 2001; 95:190-198
5. Stupp R, Mason WP, van den Bent MJ, et al. radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005; 352:987-996
6. Athanassiou H, Synodinou M, Maragoudakis E, et al. Randomized Phase II Study of temozolomide and radiotherapy compared with radiotherapy alone in newly diagnosed glioblastoma multiforme. J Clin Oncol 2005; 23(10):2372-2377
7. Hou LC, Anand Veeravagu BS, Ssu AR, et al. Recurrent Glioblastoma Multiforme: a review of natural history and management options. Neurosurg Focus (2006) 20 (4):E3
8. Gatcombe H. Advances in the management of glioblastoma multiforme: an expert interview with Dr Renato La Rocca from 2006 SNO. Medscape hematology-Oncology. 2007;10(1). Jan 2007 www.medscape.com
9. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003; 63:5821-5828
10. Singh SK, Clarke ID, Hide T, et al. Cancer stem cells in nervous system tumors. Oncogene. 2004; 23(43):7267-73
11. Al-Hajj M, Becker MW, Wicha M, et al. Therapeutic implications of cancer stem cells. Curr Opin Genet Dev. 2004;14(1):43-7
12. Donnenberg VS, Donnenberg AD. Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis. J Clin Pharmacol. 2005;45(8):872-7
13. Liu G, Yuang X, Zeng Z, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. Molecular Cancer 2006; 5:67
14. Galderisi U, Cipollaro M, Giordano A. Stem cells and brain cancer. Cell Death Differ. 2006;13(1):5-11
15. Chen L, Shen R, Ye Y, et al. Precancerous Stem Cells Have the potential for both Benign and Malignant Differentiation. PLoS One, 2007, 2(3): e293 1-16 doi: 10.1371/Journal.pone.0000293
16. Naganuma H, Sasaki A, Satoh E, et al. Transforming growth factor-_ inhibits interferon-g secretion by lymphokine-activated killer cells stimulated with tumor cells. Neurol Med Chir 1996; 36:789-795
17. Fakhrai H, Gramatikova S, Safaei R. Downregulating of Transforming Growth Factor b as Therapeutic Approach for Brain Tumors. Brain Tumor Immunotherapy. Editors Liau LM, Becker DP, Cloughesy TF, Bigner DD. Humana Press, 2001; Chap 14: 289-299
18. Parney IF, Hao C, Petruk KC. Glioma immunology and immunotherapy. Neurosurgery 2000;46(4):778-91
19. Tran CT, Wolz P, Egensperger R, et al. Differential expression of MHC class II molecules by microglia and neoplasic astroglia: relevance for the escape of astrocytoma cells from immune surveillance. Neuropathol Appl neurobiol 1998; 24(4):293-301
20. Wheeler CJ, Black KL, Liu G, et al. Thymic CD8+ T cell production strongly influences tumor antigen recognition and age-dependent glioma mortality. J. Immunol. 2003; 171:4927-4933
21. Wiesner SM, Freese A, Ohlfest JR. Emerging concepts in glioma biology: implications for clinical protocols and rational treatment strategies. Neurosurg Focus. 2005;19(4):E3
22. Westarp ME, Westarp MP, Grundl W, et al. Improving medical approaches to primary CNS malignancies-retinoid therapy and more. Med Hypotheses. 1993; 41(3):267-76
23. Kuan CT, Wikstrand CJ, Bigner DD. EGF mutant receptor VIII as a molecular target in cancer therapy. Endocr Relat Cancer. 2001; 8 (2):83-96
24. Yang W, Barth RF, Wu G, et al. Development of a syngeneic rat brain tumor model expressing EGFR VIII and its use for molecular targeting studies with monoclonal antibody L8A4. Clin Cancer Res. 2005; 11(1):341-50
25. Klatzmann D, Philippon J, Valery C, et al. Gene therapy for glioblastoma in adult patients: safety and efficacy evaluation of an in situ injection of recombinant retroviruses producing cells carrying the thymidine kinase gene of the herpes simplex type 1 virus, to be followed with the administration of glancicovir. Human Gene Therapy 1996; 7:109-126
26. van Herpen CML, De Mulder PH. Loco regional immunotherapy in cancer patients: review of clinical studies. Ann Oncol 2000;11(10):1229-39
27. Tse VCK, Conley FK. Immunostimulation and Immunomodulation of Brain Tumors. Brain Tumor Immunotherapy. Edited by Liau LM, Becker DP, Cloughesy TF, Bigner DD. Humana Press, 2001. Chap. 4:91-99
28. Fenstermaker R, Ciesielski M. Immunotherapeutic strategies for malignant glioma. Cancer Control 2004; 11(3):181-191
29. Fujimiya Y, Suzuki , Katakura R, et al. Injury to autologous normal tissues and tumors mediated by lymphokine-activated killer (LAK) cells generated in Vitro from peripheral blood mononuclear cells of glioblastoma patients. J Hematother 1999;8(1):29-37
30. Merchant RE, Ellison MD, Young HF. Immunotherapy for malignant glioma using human recombinant interleukine-2 and activated autologous lymphocytes. A review of pre-clinical and clinical investigations. J Neurooncol 1990;8(2):173-88
31. Merchant RE, Baldwin NG, Rice CD, et al. Adoptive immunotherapy of malignant glioma using tumor-sensitized T lymphocytes. Neurol Res 1997;19(2):145-52
32. Plautz G, Barnett G, Miller D, et al. Systemic T cell adoptive immunotherapy of malignant gliomas. J Neurosurg 1998; 89:42-51
33. Jean W, Spellman S, Wallenfriedman M, et al. Interleukine-12-based immunotherapy against rat 9L glioma. Neurosurgery 1998; 42(4): 850-857
34. Wallenfriedman MA, Conrad JA, Della Barre L, et al. Effects of continuous localized infusion of granulocyte-macrophage colony-stimulating factor and inoculations of irradiated glioma cells on tumor regression. J Neurosurg. 1999; 90(6):1064-71
35. Graf M, Jadus M, Hiserodt J, et al. Development of systemic immunity of glioblastoma multiforme using tumor cells genetically engineered to express the membrane-associated isoform of macrophage colony-stimulating factor. J Immunol 1999; 163:5544-5551
36. Siesjo P, Visse E, Sjogren HO. Cure of established, intracerebral rat gliomas induced by therapeutic immunizations with tumor cells and purified APC or adjuvant IFN-gamma treatment. J Immunother Emphasis Tumor Immunol. 1996; 19(5): 334-45
37. Okada H, Pollack IF, Lieberman F, et al. Gene therapy of malignant gliomas: a pilot study of vaccination with irradiated autologous glioma and dendritic cells admixed with IL-4 transduced fibroblasts to elicit an immune response. Hum Gene Ther. 2001;12(5):575-95
38. Pellegatta S, Poliani PL, Corno D, et al. Dendritic cells pulsed with glioma lysate induce immunity against syngeneic intracranial gliomas and increase survival of tumor-bearing mice. Neurol Res 2006; 28(5):527-31
39. Yu JS, Wheeler CJ, Zeltzer PM, et al. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. Cancer Res 2001;61(3):842-7
40. Okada H, Tahara H, Shurin MR, et al. Bone marrow-derived dendritic cells pulsed with a tumor-specific peptide elicit effective anti-tumor immunity against intracranial neoplasms. Int. J Cancer. 1998; 78 (2): 196-201
41. Ashley DM, Faiola B, Nair S, et al. Bone marrow-generated dendritic cells pulsed with tumor extracts or tumor RNA induce antitumor immunity against central nervous system tumors. J Exp Med. 1997; 186(7): 1177-82
42. Liau LM, Prins RM, Kiertscher SM, et al. Dendritic cell vaccination in glioblastoma patients induces systemic and intracranial T- cell responses modulated by the local central nervous system tumor microenvironment. Clin Cancer Res. 2005; K 11 (15): 5515-25
43. Kikuchi T, Akasaki Y, Irie M, et al. Results of a phase I clinical trial of vaccination of glioma patients with fusions of dendritic and glioma cells. Cancer Immunol Immunother 2001;50(7):337-44
44. Chang KJ, Nguyen PT, Thompson JA, et al. Phase I clinical trial of allogeneic mixed lymphocyte culture (cytoimplant) delivered by endoscopic ultrasound-guided fine-needle injection in patients with advanced pancreatic carcinoma. Cancer. 2000;88(6):1325-35
45. Guo Y, Wu M, Chen H, et al. Effective Tumor Vaccine Generated by Fusion of Hepatoma Cells with Activated B Cells. Science, 1993; 263: 518-520
46. Moviglia GA, Development of tumor B-cell lymphocyte hybridoma autovaccination. Results of phase I-II clinical trial. Transfusion Sci 1996 17: 643-649
47. Moviglia GA, Gaeta C, Iraola N, et al. Combination Immunotherapy for Pancreatic Cancer. Proceeding of American Society for Clinical Oncology 2001; 20: 264[a]
48. Moviglia GA, Gaeta CA, Abdelnur R, et al. Combination immunotherapy for metastatic breast cancer. Proceeding of American Society for Clinical Oncology 2003, 22: 182
49. Oken, M.M., Creech, R.H., Tormey, D.C., et al. Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 1982, 5:649-655
50. Moviglia GA, Lara P, Varela G, et al. Ensayo de proliferación linfocitaria para el monitoreo inmunológico del paciente oncológico y su utilidad en el seguimiento de los tratamientos inmunomoduladores. Archivos Argentinos de Alergia e Inmunología Clínica 1999; 30 (2): 40